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FORM PTO-1390 (Rev. 9-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 004101-003

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5): 10/009198 UNASSIGNED

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/01612	9 JUNE 2000	11 JUNE 1999

TITLE OF INVENTION
PHARMACEUTICAL COMPOSITION COMPRISING NO OR AT LEAST A NO DONOR COMPOUND OR ANOTHER COMPOUND CAPABLE OF RELEASING OR INDUCING NO FORMATION IN CELLS

APPLICANT(S) FOR DO/EO/US
Maurice ISRAEL et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:

Nine (2) Sheets of Drawings; Form PCT/IB/308 and International Preliminary Exam Report (Form PCT/IPEA/409).

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U.S. APPLICATION NO (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
UNASSIGNED 10/009198	PCT/FR00/01612	004101-003

21. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,040.00 (960)		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00 (970)		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $740.00 (958)		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00 (956)		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00 (962)		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of $130.00 (154) for furnishing the oath or declaration later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	9 -20 =	0	X$18.00 (966)	$ 0.00	
Independent Claims	2 -3 =	0	X$84.00 (964)	$ 0.00	
Multiple dependent claim(s) (if applicable)			+$280.00 (968)	$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				$ 890.00	
Reduction for 1/2 for filing by small entity, if applicable (see below).			+	$ 445.00	-
SUBTOTAL =				$ 445.00	
Processing fee of $130.00 (156) for furnishing the English translation later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$	
TOTAL NATIONAL FEE =				$ 445.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 (581) per property			+	$	
TOTAL FEES ENCLOSED =				$ 445.00	
				Amount to be refunded:	$
				charged:	$

a. ☒ Small entity status is hereby claimed.

b. ☒ A check in the amount of $ 445.00 to cover the above fees is enclosed.

c. ☐ Please charge my Deposit Account No. 02-4800 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

d. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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(81) États désignés *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Publiée:
— *Sans rapport de recherche internationale, sera republiée dès réception de ce rapport.*

En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

(54) Title: PHARMACEUTICAL COMPOSITION COMPRISING NO OR AT LEAST A NO DONOR COMPOUND OR ANOTHER COMPOUND CAPABLE OF RELEASING OR INDUCING NO FORMATION IN CELLS

(54) Titre: COMPOSITION PHARMACEUTIQUE COMPRENANT DU NO OU AU MOINS UN COMPOSE DONNEUR DE NO OU ENCORE UN COMPOSE CAPABLE DE LIBERER OU D'INDUIRE LA FORMATION DE NO DANS LES CELLULES

(57) Abstract: The invention concerns the use of NO, a NO donor compound or a compound capable or releasing, stimulating or inducing NO formation in cells to prepare a medicine for treating or preventing a disease resulting from deficiency of an adult gene in a person for the re-expression of said homologous foetal gene. The invention particularly concerns the treatment of Duchenne or Becker muscular dystrophy, or thalassemia or sickle cell disease.

(57) Abrégé: La présente invention concerne l'utilisation de NO, d'un composé donneur de NO ou d'un composé capable de libérer, de favoriser ou d'induire la formation de NO dans les cellules pour la préparation d'un médicament destiné au traitement ou à la prévention d'une maladie résultant de la déficience d'un gène adulte chez un individu par la ré-expression dudit gène foetale homologue. La présente invention concerne tout particulièrement le traitement des dystrophies musculaires, comme la dystrophie musculaire de Duchenne ou de Becker, ou la thalassémie ou la drépanocytose.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Maurice ISRAEL et al.	Group Art Unit: (Unassigned)
Application No.: Unassigned (Corresponds to PCT/FR00/01612)	Examiner: (Unassigned)
International Filing Date: June 9, 2000	
For: PHARMACEUTICAL COMPOSITION COMPRISING NO OR AT LEAST A NO DONOR COMPOUND OR ANOTHER COMPOUND CAPABLE OF RELEASING OR INDUCING NO FORMATION IN CELLS	

PRELIMINARY AMENDMENT

BOX PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination, please amend the above-captioned application as follows:

IN THE CLAIMS:

Kindly amend claims 1-8 and add new claim 9 as follows:

1. (Amended) A method for the treatment or prevention of a disease resulting from deficiency of an adult gene in an individual through the re-expression of said homologous foetal gene, said method comprising using an effective amount of NO, a NO donor compound or a compound able to release, promote or induce NO formation cells.

2. (Amended) The method according to claim 1, which is intended to reactivate the expression of at least one foetal gene in adult tissues such as to restore the presence and/or the localization of at least one foetal protein.

3. (Amended) The method according to claim 1, wherein the foetal gene codes for the embryonic form of the protein encoded by the deficient gene.

4. (Amended) The method according to claim 1, wherein the compound able to induce NO formation is L-arginine, or one of its derivatives, forming a substrate for NO-synthase or promoting availability of the substrate.

5. (Amended) The method according to claim 1, wherein the definite gene is the dystrophin gene and the foetal gene is the utrophin gene.

6. (Amended) The method according to claim 1, wherein the deficient gene is the haemoglobin gene and the foetal gene is the foetal haemoglobin gene.

7. (Amended) The method according to claim 1, wherein the disease resulting from the deficiency of an adult gene is a muscular dystrophy, thalassaemia or sickle-cell disease.

8. (Amended) Pharmaceutical composition comprising NO and/or at least one NO donor or a compound able to release, promote or induce NO formation in cells, associated in said composition with a pharmaceutically acceptable vehicle.

--9. The method according to claim 7, wherein the muscular dystrophy is Duchenne or Becker muscular dystrophy. --

REMARKS

Entry of the foregoing amendment(s) is respectfully requested.

The claims have been amended to eliminate multiple dependency and to place them in better condition for U.S. patent practice.

Should the Examiner have any questions concerning the subject application, a telephone call to the undersigned would be appreciated.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: _____
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Date: December 10, 2001

Application No. (Unassigned)
Attorney's Docket No. 004101-003

Attachment to Preliminary Amendment dated December 10, 2001

Marked-up Claims 1-8

1. (Amended) [Use of NO, of a NO donor compound or of a compound able to release, promote or induce NO formation in cells, to prepare a medicinal product intended] A method for the treatment or prevention of a disease resulting from deficiency of an adult gene in an individual through the re-expression of said homologous foetal gene, said method comprising using an effective amount of NO, a NO donor compound or a compound able to release, promote or induce NO formation cells.

2. (Amended) [Use of NO or of a No donor compound or compound able to release, promote or induce NO formation in cells according to claim 1, characterized in that said medicinal product] The method according to claim 1, which is intended to reactivate the expression of at least one foetal gene in adult tissues such as to restore the presence and/or the localization of at least one foetal protein.

3. (Amended) [Use according to either of claims 1 or 2] The method according to claim 1, [characterized in that] wherein the foetal gene codes for the embryonic form of the protein encoded by the deficient gene.

4. (Amended) [Use according to any of claims 1 to 3] The method according to claim 1, [characterized in that] wherein the compound able to induce NO formation is L-arginine, or one of its derivatives, forming a substrate for NO-synthase or promoting availability of the substrate.

Attachment to Preliminary Amendment dated December 10, 2001

Marked-up Claims 1-8

5. (Amended) [Use according to any of the preceding claims] The method according to claim 1, [characterized in that] wherein the definite gene is the dystrophin gene and the foetal gene is the utrophin gene.

6. (Amended) [Use according to any of the preceding claims] The method according to claim 1, [characterized in that] wherein the deficient gene is the haemoglobin gene and the foetal gene is the foetal haemoglobin gene.

7. (Amended) [Use according to any of the preceding claims] The method according to claim 1, [characterized in that] wherein the disease resulting from the deficiency of an adult gene is a muscular dystrophy, [such as Duchenne or Becker muscular dystrophy, or] thalassaemia or sickle-cell disease.

8. (Amended) Pharmaceutical composition [characterized in that it contains] comprising NO and/or at least one NO donor or a compound able to release, promote or induce NO formation in cells, associated in said composition with a pharmaceutically acceptable vehicle.

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PHARMACEUTICAL COMPOSITION COMPRISING NO OR AT LEAST A NO DONOR COMPOUND OR ANOTHER COMPOUND CAPABLE OF RELEASING OR INDUCING NO FORMATION IN CELLS

The subject of the invention is a pharmaceutical composition containing NO or at least a compound able to release or induce NO formation in cells, such as a NO donor or a NO synthase substrate, to re-express a foetal protein whose adult isoform is muted and/or absent. The invention is therefore of interest for the treatment of diseases in which the adult gene is deficient or absent. By way of example, utrophin, the foetal homologous form of dystrophin, may replace the latter in Duchenne and Becker myopathies. Similarly, foetal haemoglobin may replace the adult haemoglobin in thalassaemia and sickle-cell disease. The present invention is therefore remarkable in that it provides the possibility of replacing current methods of treatment of these pathologies by use of the NO route to activate expression of the foetal protein. The invention particularly concerns the use of NO, of a NO donor or of a compound able to release or induce NO formation in the cells to prepare a medicinal product intended for the

treatment or prevention of Duchenne and Becker myopathies and of thalassaemia and sickle-cell disease.

The work conducted on sickle-cell disease and thalassaemia has demonstrated that hydroxyurea and butyrate are able to reactivate the expression of the foetal gene of haemoglobin. This result could be explained by common metabolic phenomena. The urea cycle and the Krebs cycle are coupled together and if hydroxy-urea interferes with the urea cycle, it could lead to retro-regulation of the Krebs cycle, which would cause a lower consumption of acetyl-CoA and therefore the formation of ketone bodies such as beta-hydroxybutyrate.

The metabolic phenomena associated with the expression of foetal genes relate to a low oxidizing metabolism and high glycolysis. Consequently, the histochemical analysis of foetal muscle fibres has shown that the glycolytic enzymes are more expressed than the oxydizing enzymes. In addition, it has been shown that the mode of nitrogen secretion in the embryo is more ammonotelic than ureotelic, corresponding to slowed functioning of the urea cycle. Under these conditions, L-arginine, which is an essential substrate for the urea cycle, is deviated towards other routes such as the NO-synthase (NOS) or amidinotransferase routes, hence leading to an increase in nitric oxide and creatine levels in the embryo.

The understanding of these metabolic phenomena has led the inventors to reproducing this metabolic situation in adult animals and in cultured cell systems in order to demonstrate that the use of L-arginine and NO enables the reactivation of foetal genes in adult tissues such that

the presence and localisation of foetal proteins can be restored.

The work which led to the present invention was conducted for the purpose of treating patients suffering from Duchenne and Becker myopathies, or from thalassaemia and sickle-cell disease, using this new foetal gene reactivation strategy; but the understanding of the metabolic phenomena described above can be used to transpose the latter to the treatment of any disease in which the deficient adult gene has a foetal homologue.

Duchenne muscular dystrophy, hereinafter called DMD, is a genetic disease related to chromosome X, in which lack of a protein of the membrane cytoskeleton is observed, dystrophin, leading to progressive muscle wasting. Three types of DMD treatment are currently being considered: pharmacological treatment with gluco-corticoids, myoblast transplant and gene therapy (10). It has also been suggested to offset the loss of dystrophin by reactivating the expression of utrophin. It would seem, in effect, that utrophin is able to perform the same cell functions as dystrophin and would therefore be able to compensate for the absence of dystrophin (3,7). Utrophin is found in the muscles in both MSD patients and in controls (24). Although the utrophin gene in adults is not fully extinguished, utrophin is considered to be the foetal homologue of dystrophin. The difference in adults is its localisation; it is no longer found in the sarcolemma, where it is replaced by dystrophin, but it persists in satellite cells, the neuromuscular junctions and the capillaries (20) where NO-synthase (NOS) is particularly abundant. Among the different isoforms of

NOS, there exists a specific muscle form, NOS-mu, which is an isoform derived from alternate splicing, having catalytic activity which is equivalent to that of the neuronal isoform (34). NOS has been found in the sarcolemma of both the fast and slow contraction fibres (17, 31). In mdx mice, an animal model of DMD, NOS is not anchored in the sarcolemma but is delocalised inside the muscle fibres (5). Also, it has been recently demonstrated that NOS localisation is restored after transfection of the dystrophin gene in the muscles of mdx mice (9). This would suggest the participation of this enzyme or its product in the assembly of the protein complex present underneath the sarcolemma.. Having regard to these observations, the inventors have evidenced the possibility of using NO to re-express utrophin, foetal haemoglobin or other foetal proteins. In the prior art the use of vasodilators, such as hot baths, was put forward but the effect of NO or of a NO donor compound on the re-expression of utrophin and foetal haemoglobin has never been described.

The work conducted under the present invention has shown that in cultured myotubes L-arginine and NO donor compounds increase both the level and the membrane localisation of utrophin. After injection of L-arginine in the muscles, the localisation of utrophin at the membrane of the muscle fibre occurs in control mice and increases in mdx mice (which show natural, low over-expression).

The mechanisms which lead to the expression and localisation of utrophin at the sarcolemma are not clear. No could be able to nitrate the tyrosines of some transcriptional factors which are normally phosphorylated

thereby promoting the expression of utrophin in the myotubes and its addressing towards the membrane. Another explanation could be that NO acts via the production of cGMP as suggested by its reduced action in the presence of OQD, a selective inhibitor of guanylate cyclase. The degradation products of L-arginine could therefore control the complex organisation of the proteins under the membrane of the muscle fibre.

The mRNA of utrophin in the muscle was observed throughout the sarcolemma, with preferential expression at the neuromuscular junction (14, 40). Up until now, two molecules expressed at the neuromuscular junction, neural agrin and heregulin, have been identified as being respectively capable of increasing the expression of utrophin in the cytoplasm (15) and mRNA levels of utrophin (16). But the possibility of using these molecules in the treatment of DMD remains to be shown.

The purpose of the present invention is therefore to offer a new treatment strategy for diseases resulting from deficiency of an adult gene by restoring the activity of a foetal gene homologous to said adult gene.

This purpose is achieved through the use of NO, a NO donor compound or a compound able to release, induce or promote NO formation in the cells, to prepare a medicinal product intended for the treatment or prevention of a disease resulting from the deficiency of an adult gene in a patient having a foetal gene homologous to said adult gene by means of the re-expression of the homologous foetal gene if such exists.

The treatment method of the invention may be used in lieu and stead of hydroxyurea or butyrate for example in cases of thalassaemia and sickle-cell disease.

By compound able to release or induce NO formation is meant any compounds such as NO donors or compounds able to promote NO formation in cells.

More particularly, the invention concerns the use of NO, of a NO donor compound or of a compound able to release, promote or induce NO formation in cells, to prepare a medicinal product intended to reactivate the expression of at least one foetal gene in adult tissues such as to restore the presence and/or localisation of at least one foetal protein.

The use according to the invention makes it possible to reactivate the foetal situation by re-expressing the embryonic form of the protein encoded by the deficient gene.

Some compounds such as hydroxyurea or beta-hydroxybutyrate are toxic or ill-tolerated, therefore the invention more particularly concerns, as compound able to induce NO formation, either L-arginine or its derivatives such as hydroxy-arginine or its boron derivatives which promote NO production or substrate preservation. In one preferred embodiment of the invention, L-arginine is administered in the proportion of 200 mg/kg for 3 to 4 weeks.

But the invention more largely concerns the use of NO donors or compounds involved in metabolic pathways enabling an increase in the cell production of NO.

It is known that Duchenne and Becker dystrophies are connected with the deletion or mutation of a gene of

chromosome X. Therefore, dystrophin is an essential protein in muscle function, whose absence or mutation leads to muscle degeneration. The disease evolves gradually as the muscle degenerates owing to the absence of dystrophin. The present invention sets out specifically to reactivate the embryonic protein, namely utrophin, to treat or prevent DMD. The work conducted under the present invention has shown that the injection of a pharmaceutical composition containing NO or at least a NO donor compound or a compound able to release, promote or induce NO formation in the cells, makes it possible to induce the onset of utrophin at the sarcolemma of dystrophic and normal muscles in *vitro* on myotube cultures. Similarly, *in vivo* it was observed that the injection of said composition in mice leads to major expression of utrophin at the sarcolemma.

Consequently, the invention especially concerns the use of NO and/or at least a NO donor compound or a compound able to release, promote or induce NO formation in cells, to prepare a medicinal product for the re-expression of the foetal protein as a spare wheel for the deficient adult protein. More particularly, with the method of the invention, it is possible to reactivate the expression of utrophin in adult tissues such as to restore the presence and localisation of this protein at the sarcolemma, so that utrophin replaces dystrophin, whenever the latter is absent.

The invention therefore also concerns a pharmaceutical composition containing NO or at least a NO donor compound or a compound able to release, promote or induce NO formation in the cells, associated in said

composition with a pharmaceutically acceptable vehicle for per os, cutaneous, intraperitoneal, intravenous or subcutaneous administration.

Other advantages and characteristics of the invention will become apparent on reading the following description describing the work conducted on DMD within the scope of this invention.

The most frequent DMD (11) (1 out of 3500 boys) and the most severe myopathy is characterized by gradual loss of muscular strength, finally leading to marked fibrosis and fatty infiltration. The DMD gene (25) spans approximately 2300 kb on band p21, and most DMD mutations are intragene deletions, leading to the absence of dystrophin, a protein of 427 kD, in patient muscle (18, 1). Dystrophin is a large protein of the cytoskeleton localised on the inner surface of the sarcolemma of normal muscle. Dystrophin is associated with a complex of glycoproteins and membrane proteins respectively called DAGs for "dystrophin-associated glycoproteins" and DAPs for "dystrophin-associated proteins" which are considerably reduced in the muscle of patients suffering from DMD (2, 28). One of the proteins, syntrophin, is associated with NOS via a PDZ domain (4). The dystrophin-glycoprotein complex binds the subsarcolemmal cytoskeleton to the extracellular matrix. Dystrophin is involved in maintaining the morphological and functional structure of striated muscle fibre and in calcium homeostasis.

An autosomal transcript of 13 kb encoded by a gene of the long arm of chromosome 6 in man and chromosome 10 in mice, has been identified. It encodes a protein having more than 80 % homology to dystrophin, called utrophin, of

395 kD (23, 36). The homology between dystrophin and utrophin extends along their entire length suggesting that they derive from a common ancestral gene. Utrophin, like dystrophin, binds to actine via the N-terminal domain, and C-terminal domain is highly conserved. Utrophin is associated with a complex of sarcolemmal proteins that are identical or at least antigenically similar to those of dystrophin. Its localisation is the same as that of the acetycholine receptor, at the top of the post-synaptic folds. Utrophin is perhaps one of the molecules of the cytoskeleton which organizes and stabilizes the cytoplasmic domain of the acetylcholine receptor.

Patients suffering from DMD and Becker dystrophy (a less severe form of DMD) and mdx mice maintain some expression of utrophin at the sarcolemma (35, 20, 21, 24) probably to compensate for the absence of dystrophin. The methods for post-regulating expression of the utrophin gene are beneficial to muscle function. For example, the use of the transgenic expression firstly of truncated utrophin and then of full-length utrophin in mice led to demonstrating that utrophin can functionally replace dystrophin (8, 38, 39): the overexpression of utrophin leads to the restoration of all the components of DAGs, and muscle performance is increased. The overexpression of utrophin saves the deterioration of the diaphragm, the most severely affected muscle in mdx mice. Also utrophin-deficient mice show a phenotype of slight myopathy, like mdx mice with dystrophin deficiency, but mice with both dystrophin and utrophin deficiency show severe myopathy of the skeletal and cardiac muscles (33). The expression of a transgene of truncated utrophin in the muscles of mice

with both dystrophin and utrophin deficiency, gives protection against death and the development of any clinical phenotype (30).

During the development stage, utrophin is found on the membrane surface of immature fibres in normal embryos and is gradually replaced by dystrophin, except at the neuromuscular junction where it persists (26). Therefore, it is possible to consider utrophin as the foetal homologue of dystrophin (36). Several observations have brought to light the mechanism which governs the changeover from the foetal gene to the adult gene. Patients suffering from sickle-cell disease or thalassaemia who have an abnormal adult haemoglobin gene, were treated with butyrate or hydroxyurea which reactivated the foetal haemoglobin gene (32, 29, 27). It is possible to expect a high level of glycolysis in the foetus (12, 6) with preferential movement of acetyl-CoA towards the anabolic routes. Low oxydizing phosphorylation should promote acetyl-CoA pathways to the ketone bodies. The subsequent accumulation of beta-hydroxybutyrate could then induce the expression of the foetal genes. Since the Krebs cycle and the urea cycle are coupled, low oxydizing phosphorylation is correlated with low urea production, which may also be induced by treatment with hydroxyurea. This could result in high levels of L-arginine which could therefore be used as substrate for NOS and amidinotransferase leading to creatine. Nitric oxide (NO) would then give the signal for the expression of foetal genes which would therefore be responsible for the high levels of creatine found in the urine of patients suffering from DMD. The mechanisms envisaged above by the

inventors led them to testing the effects of L-arginine and NO donor compounds on the expression of utrophin. The inventors were therefore able to show in remarkable manner that in normal adult mice and in mdx mice treated chronically with L-arginine, which is a substrate of NOS, the levels of muscle utrophin increased at the membrane along the entire length of the sarcolemma. The experiments reported below show in surprising manner that the treatment of NO donors with L-arginine increases the levels of utrophin and its membrane localisation in normal and mdx cultured myotubes. Similar results were obtained with hydroxyurea which was used as a control, as it is known that this product activates foetal haemoglobin.

Method

1) Treatment of mice

Three normal, adult mice aged 18 months (C57 BL/6 line) and three mdx mice were given a daily intraperitoneal injection of 200 mg/kg L-arginine for three weeks. Two other groups of three adult mice were used as controls and were given a daily injection of physiological serum.

The mice were sacrificed by ether anaesthesia, the biceps femoris and the semi-tendinous muscles were quickly dissected from the hind limbs of each animal and frozen in liquid nitrogen.

2) Cell culture

Myotubes were obtained from a normal cell line (NXLT) and a mdx cell line as described by Liberona *et al* (22), and C2 myotubes as described by Inestrosa *et al* (19).

3) Immunofluorescence

In vivo. After cold fixing in methanol (-20°C for 10 minutes) sections of 7 µm were incubated for two hours with a utrophin specific monoclonal antibody (NCL-DRP 2, Novacastra) (/10 vol/vol) in PBS containing 0.1 % saponin and 0.2 % bovine albumin. The second antibody labelled with fluoroscein (N 1031, Amersham) was diluted (1/4000 vol/vol) in PBS containing 0.1 % saponin and incubated for one hour.

In vitro. The cultures were treated as described previously with the exception of the second antibody labelled with fluoroscein which was diluted to 1/100 vol/vol. The incubation time was 2 hours for the first and second antibody.

4) Immunoblotting

The myotubes obtained from the NXLT, XLT and C2 lines were homogenized using a Polytron (Kinematica) in 10 mM Tris-HCl pH 6.8, 1 % Triton X-100, 1% SDS, 0.5 % sodium deoxycholate on ice. The quantity of total proteins was determined following the protocol for the bicinchoninic acid protein test (BCA, Pierce). Equivalent quantities of protein were separated by SDS-Page on 5% gel, then electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell). The membranes were then incubated with the same monoclonal antibody directed against utrophin used for the immunofluorescence techniques (1/250 vol/vol). The fixed antibodies were detected with a Sanofi anti-mouse goat secondary antibody (1/5000 vol/vol) bound to horseradish peroxydase and developed by

chemioluminescence reaction (ECL, Amersham Pharmacy Biotech).

Results

In the results given below, reference is made to the appended illustrations in which:

- Figure 1 shows the occurrence of utrophin under the sarcolemma of normal adult mice and mdx mice chronically treated with L-arginine (magnification X 300). Figure 1 shows the immunolocalisation of utrophin on the muscle membrane of normal mice and mdx mice treated with L-arginine. (a) control corresponding to normal mice given an injection of physiological serum: no utrophin observed at the sarcolemma. (b) normal mice treated with the L-arginine: utrophin is seen under the sarcolemma. (c) control corresponding to the mdx mice given an injection of physiological serum: utrophin is visible at the sarcolemma. (d) mdx mice given L-arginine: increase in utrophin levels under the sarcolemma.
- Figure 2 shows the variation of utrophin in the myotubes after treatment involving nitric oxide (NO) (magnification x 200). A, a-h: normal cell line (NXTL). B, a'h': mdx cell line (XLT). The cell cultures were treated by exposure of the differentiated myotubes to drugs for 48 hours. A, a': control cultures. B,b': L-arginine (2.10^{-3} M). c c': SIN-1 (10^{-3} M). d, d': SIN-1 (10^{-3} M) + L-arginine (10^{-3} M). e, e': D-arginine (10^{-3} M). f, f': L-arginine (10^{-3} M) + OQD (10^{-5} M). g,g': L-NMMA (10^{-3} M). h, h': hydroxyurea (10^{-4} M).

Figure 3 shows the increase in utrophin levels in NXLT, XLT and CT myotubes under the action of L-arginine.

Immunoblot analysis of utrophin was conducted under control conditions (CTRL) and after 48 hours' treatment with 2.10^{-3} M L-arginine (L-arg).

The adult mice given an intraperitoneal injection of L-arginine for three weeks were sacrificed. After sacrifice, the thigh muscles were prepared by immunocytochemistry. After this treatment, utrophin was detected underneath the sarcolemma in the muscle fibres of normal mice as shown in figure 1a. Treatment of mdx mice with L-arginine increased the utrophin level already present in the sarcolemma (35, 21). Both in normal mice and in mdx mice, immunolabelling covers the sarcolemma and is present on part of the interstitial tissue. This labelling is probably due to the utrophin expressed by the capillaries and satellite cells.

This effect of arginine was then examined on cultured myotubes which are more suitable for direct application of drugs and avoids interference with non-muscular utrophin. The NXLT and XLT myotubes of normal and mdx mice respectively were used for immunochemical testing of the effects of L-arginine and NO on the expression of utrophin. After 48 hours' treatment, utrophin labelling increased when the synthesis of endogenous NO was increased via excess L-arginine and when SIN-1 was applied as shown in figure 2. Utrophin was co-localized with the large clusters of acetylcholine receptors present on the myotubes evidencing that part of the labelling is membrane-related (not shown in the appended figure). The increased labelling of utrophin was also observed to a lesser extent on the cells of C2 mice myotubes and primary rat myotubes. The accumulated application of SIN-1 and L-

arginine further increases utrophin labelling as shown in figure 2. The absence of any effect by D-arginine illustrated in figure 2 demonstrates the involvement of NO in the method of the invention. The basal level of utrophin in the absence of NO-synthase activity shown in figure 2 was obtained after application of N^G-methyl-L-arginine (L-NMMA) which is an inhibitor of NOS. It is widely acknowledged that the intracellular effects of NO are mediated through the activation of soluble guanylate cyclase. The synthesis of utrophin induced by NO was inhibited in the presence of ODQ (13) which is an antagonist specific to guanylate cyclase as shown in figure 2. Figure 2 also shows that the hydroxyurea used by analogy with the treatment of thalassaemia, also increases utrophin labelling in remarkable manner. This effect probably arises from action on the expression of utrophin.

In order to complete the analysis of the effect of NO production on utrophin expression in normal and mdx mice, the inventors extracted the proteins from myotube cultures either treated or not treated with L-arginine under the same conditions as previously. The Western-blots in figure 3 show an evident increase of utrophin in both types of cell lines, thereby confirming imuunocytochemical data. This increase in utrophin after treatment with L-arginine was confirmed in a cell line of C2 myotubes (figure 3).

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CLAIMS

1. Use of NO, of a NO donor compound or of a compound able to release, promote or induce NO formation in cells, to prepare a medicinal product intended for the treatment or prevention of a disease resulting from deficiency of an adult gene in an individual through the re-expression of said homologous foetal gene.

2. Use of NO or of a NO donor compound or a compound able to release, promote or induce NO formation in cells according to claim 1, characterized in that said medicinal product is intended to reactivate the expression of at least one foetal gene in adult tissues such as to restore the presence and/or the localization of at least one foetal protein.

3. Use according to either of claims 1 or 2, characterized in that the foetal gene codes for the embryonic form of the protein encoded by the deficient gene.

4. Use according to any of claims 1 to 3, characterized in that the compound able to induce NO formation is L-arginine, or one of its derivatives, forming a substrate for NO-synthase or promoting availability of the substrate.

5. Use according to any of the preceding claims, characterized in that the definite gene is the dystrophin gene and the foetal gene is the utrophin gene.

6. Use according to any of the preceding claims, characterized in that the deficient gene is the haemoglobin gene and the foetal gene is the foetal haemoglobin gene.

7. Use according to any of the preceding claims, characterized in that the disease resulting from the deficiency of an adult gene is a muscular dystrophy, such as Duchenne or Becker muscular dystrophy, or thalassaemia or sickle-cell disease.

8. Pharmaceutical composition characterized in that it contains NO and/or at least one NO donor or a compound able to release, promote or induce NO formation in cells, associated in said composition with a pharmaceutically acceptable vehicle.

Fig. 1
Fig. 1 (cont.)
Fig. 2A NXLT
Fig. 2A NXLT (cont.)
Fig. 2A NXLT (cont.)
Fig. 2B XLT
Fig. 2B XLT (cont.)
Fig. 2B XLT (cont.)
Fig. 3

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En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

(54) Title: PHARMACEUTICAL COMPOSITION COMPRISING NO OR AT LEAST A NO DONOR COMPOUND OR ANOTHER COMPOUND CAPABLE OF RELEASING OR INDUCING NO FORMATION IN CELLS

(54) Titre: COMPOSITION PHARMACEUTIQUE COMPRENANT DU NO OU AU MOINS UN COMPOSE DONNEUR DE NO OU ENCORE UN COMPOSE CAPABLE DE LIBERER OU D'INDUIRE LA FORMATION DE NO DANS LES CELLULES

(57) Abstract: The invention concerns the use of NO, a NO donor compound or a compound capable or releasing, stimulating or inducing NO formation in cells to prepare a medicine for treating or preventing a disease resulting from deficiency of an adult gene in a person for the re-expression of said homologous foetal gene. The invention particularly concerns the treatment of Duchenne or Becker muscular dystrophy, or thalassemia or sickle cell disease.

(57) Abrégé: La présente invention concerne l'utilisation de NO, d'un composé donneur de NO ou d'un composé capable de libérer, de favoriser ou d'induire la formation de NO dans les cellules pour la préparation d'un médicament destiné au traitement ou à la prévention d'une maladie résultant de la déficience d'un gène adulte chez un individu par la ré-expression dudit gène foetale homologue. La présente invention concerne tout particulièrement le traitement des dystrophies musculaires, comme la dystrophie musculaire de Duchenne ou de Becker, ou la thalassémie ou la drépanocytose.

WO 00/76451 A2

Fig.1

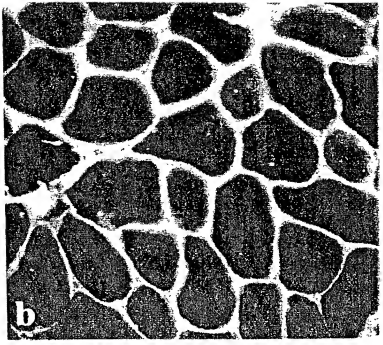
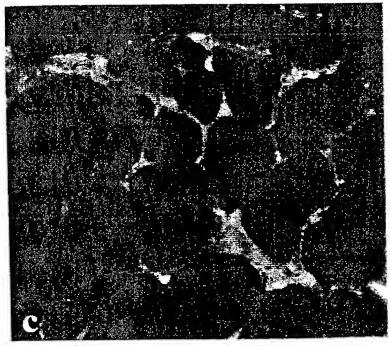


Fig.1 Suite



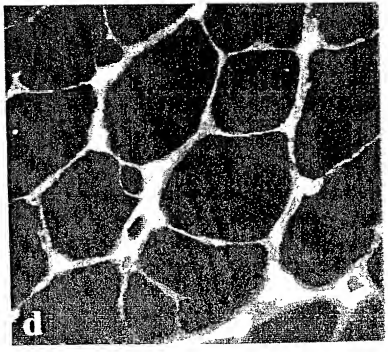


Fig.2 A NXLT

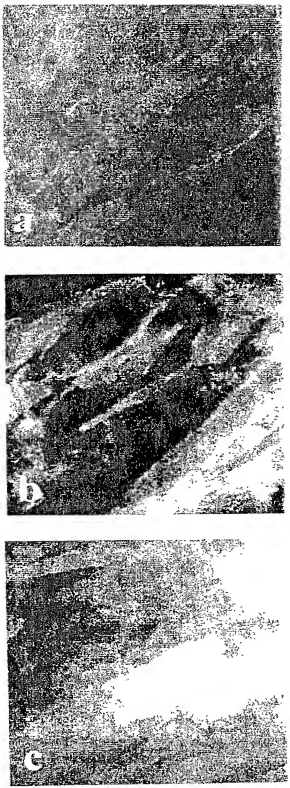


Fig.2 A NXLT Suite

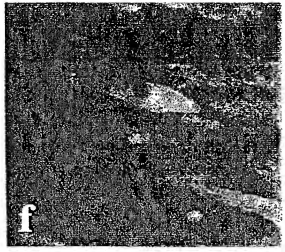


Fig.2 A NXLT Suite

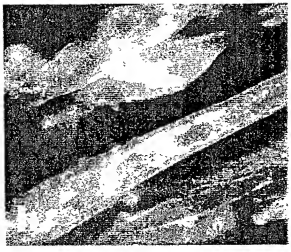


Fig.2 B XLT

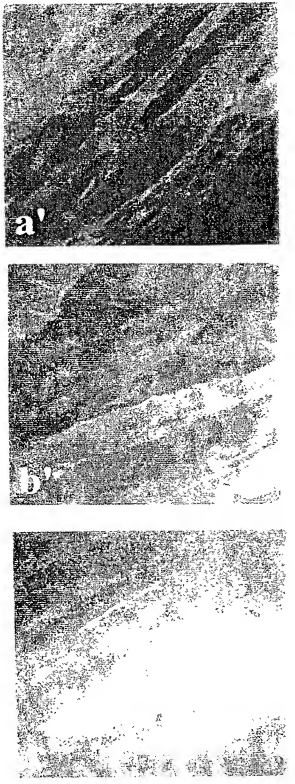


Fig.2 B XLT Surface

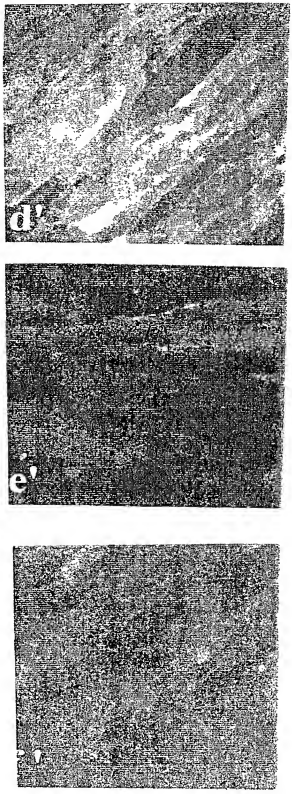


Fig.2 B X_I S_ite

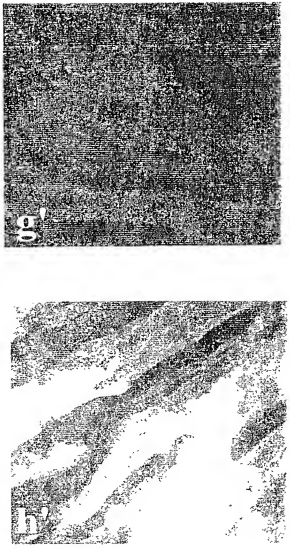


Fig.3

033525-001
Attorney's Docket No.

COMBINED DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PHARMACEUTICAL COMPOSITION COMPRISING NO OR AT LEAST A NO DONOR COMPOUND OR ANOTHER COMPOUND CAPABLE OF RELEASING OR INDUCING NO FORMATION IN CELLS

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application
Number _____ on _____
and was amended on _____ (if applicable)

☒ was filed as PCT international application
Number PCT/FR00/01612 on JUNE 9, 2000
and was amended on DECEMBER 10, 2001 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §§119 (a)-(d), 172 or 365 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §§119(a)-(d), 172 or 365:				
COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. §§119, 172 or 365	
FRANCE	99/07442	11 JUNE 1999	X Yes	No
			Yes	No
			Yes	No
			Yes	No
			Yes	No

(01/02)

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

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1-00

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Combined Declaration and Power of Attorney
for Utility or Design Patent Application
Attorney's Docket No. 033525-001
Page 3 of 3

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